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Purification and characterization of a three-component salicylate 1-hydroxylase from *Sphingomonas* CHY-1

Yves Jouanneau*, Julien Micoud, and Christine Meyer

CEA, DSV, iRTSV, Laboratoire de Chimie et Biologie des Métaux, 17 rue des Martyrs, F-38054, Grenoble, France. CNRS, UMR 5249, F-38054, Grenoble. Université Joseph Fourier, F-38000, Grenoble.

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Corresponding author:

Yves Jouanneau

LCBM/iRTSV, CEA-Grenoble

F-38054 Grenoble Cedex 9, France.

Tel. : 33 (0)4 38 78.43.10; Fax : 33 (0)4 38 78.51.85

Email : yves.jouanneau@cea.fr

Summary

In the bacterial degradation of polycyclic aromatic hydrocarbons (PAHs), salicylate hydroxylases catalyze essential reactions at the junction between the so-called upper and lower catabolic pathways. Unlike the salicylate 1-hydroxylase from pseudomonads, which is a well-characterized flavoprotein, the enzyme found in sphingomonads appears to be a three-component Fe-S protein complex, which has not so far been characterized. Here, the salicylate 1-hydroxylase from *Sphingomonas* CHY-1 has been purified and characterized with respect to its biochemical and catalytic properties. The oxygenase component called PhnII, exhibited an $\alpha_3\beta_3$ heterohexameric structure and contained one Rieske-type [2Fe-2S] cluster and one mononuclear iron per α subunit. In the presence of purified reductase (PhnA4) and ferredoxin (PhnA3) components, PhnII catalyzed the hydroxylation of salicylate to catechol with a maximal specific activity of 0.89 U/mg and showed an apparent K_m for salicylate of 1.1 ± 0.2 μM. The hydroxylase exhibited similar activity levels with methylsalicylates and low activity with salicylate analogues bearing additional hydroxyl or electron-withdrawing substituents. PhnII converted anthranilate to 2-aminophenol and exhibited a relatively low affinity for this substrate (K_m = 28 ± 6 μM). 1-Hydroxy-2-naphthoate, which is an intermediate in phenanthrene degradation, was not hydroxylated by PhnII, but induced a high rate of uncoupled oxidation of NADH. It also exerted a strong competitive inhibition of salicylate hydroxylation, with a K_i of 0.68 μM. The properties of this three-component hydroxylase are compared with those of analogous bacterial hydroxylases and discussed in the light of our current knowledge of PAH degradation by sphingomonads.

INTRODUCTION

Bacterial isolates belonging to the sphingomonad group have been shown to degrade a wide range of organic pollutants, including recalcitrant compounds such as chlorinated xenobiotics (16), dioxins (26) and polycyclic aromatic hydrocarbons (PAHs) (9, 18, 20, 32). The remarkable capabilities of this group of bacteria suggested that they might be useful for bioremediation purposes, and prompted studies on the identification of the catabolic enzymes involved. Independent genetic analyses on different PAH-degrading strains revealed that they contain a unique set of catabolic genes, often carried on a megaplasmid, which is probably responsible for their versatile metabolism (15, 20, 23). Extending over a 40-kb DNA region, the main catabolic gene cluster includes *xyl* genes involved in the catabolism of monoaromatics interspersed with genes predicted to function in the degradation of polycyclic substrates (designated as *bph, nah, ahd* or *phn*). This unique gene arrangement, which is remarkably conserved between strains of various origins, contrasts with that found in other degraders such as pseudomonads. In *Sphingobium yanoikuyae* B1, the biodegradation of naphthalene is thought to proceed through a pathway similar to that found in *Pseudomonas* species, with a first set of enzymes converting naphthalene to salicylate and a second set responsible for the conversion of salicylate to Krebs cycle intermediates (15). In this strain as in other sphingomonads, many of the gene products involved in this pathway have been identified based on sequence similarity with known *Pseudomonas* enzymes, but no counterpart was found for NahG which catalyzes the monohydroxylation of salicylate to catechol (30). In addition, the function of many genes in the catabolism of other PAHs remains to be elucidated.

Peculiar to sphingomonads is the occurrence of multiple copies of genes predicted to encode the terminal component of Rieske-type oxygenases (21, 23). Genes coding for two cognate electron carriers are generally found adjacent to such oxygenase-encoding genes in other

bacteria degrading aromatic hydrocarbons, but it is not the case in sphingomonads. Rieske-type oxygenases are known to catalyze the dihydroxylation of various aromatic compounds, and often initiate the oxidative biodegradation of pollutants. They constitute a large family of two or three component metalloenzymes the catalytic component of which is generally an heteromeric $\alpha_3\beta_3$ hexamer containing one Rieske-type [2Fe-2S] cluster and one non-heme iron atom per α subunit. The enzyme responsible for the initial attack on PAHs has recently been identified in *Sphingomonas* CHY-1, a strain able to grow on chrysene as sole carbon source (9, 25). The oxygenase, called PhnI, is functionally associated with a NAD(P)H-oxidoreductase (PhnA4) and a ferredoxin (PhnA3) in a three-component enzyme complex able to oxidize a wide range of 2- to 5-ring PAHs (13). Analysis of a knockout mutant demonstrated that PhnI was absolutely required for growth on PAHs, indicating that no other oxygenase could replace PhnI in the initial dihydroxylation of PAHs (9). Another oxygenase named PhnII, which catalyzes the C1-hydroxylation of salicylate to catechol has been identified in strain CHY-1. When overproduced in recombinant form in *E. coli*, PhnII required the co-expression of the ancillary proteins PhnA4 and PhnA3 for full activity, indicating that it is a three-component enzyme which shares electron carriers with PhnI (9). PhnII resembles salicylate 5-hydroxylase from *Ralstonia* sp. strain U2, which oxidizes salicylate to gentisate (31) but differs from salicylate 1-hydroxylases found in pseudomonads, which are monomeric flavoproteins (29). Interestingly, PhnII is also related to anthranilate dioxygenase from *Burkholderia cepacia* DBO1, a three-component enzyme that exclusively catalyses dioxygenation reactions (6). The occurrence of three-component salicylate 1-hydroxylases was first reported by Pinyakong et al., in *Sphingobium* sp. strain P2, a phenanthrene degrading strain (22). This strain synthesized three isoenzymes homologous to PhnII, which showed different specificities toward substituted salicylates. Sequence comparisons using BLAST indicated that PhnII showed substantial similarities with one

hydroxylase from strain P2 (83% and 71% identities between the α and β subunits, respectively) but only moderate similarities with the other two P2 enzymes (>51% and >45% identities). Such comparisons of protein sequences did not reveal any clue that would explain the differences in selectivity between these enzymes. In *Sphingobium yanoikuyae* B1, indirect evidence indicated that an analogous hydroxylase catalyzed both salicylate hydroxylation and conversion of 1-hydroxy-2-naphthoate to 1.2-dihydroxynaphthalene (7). The latter reaction is a key step in the biodegradation of phenanthrene by Gram negative bacteria.

So far, none of the three-component hydroxylases considered above has been purified, and therefore biochemical and catalytic data on this important class of enzymes are lacking. Here, we have purified the PhnII enzyme to near homogeneity and have investigated the kinetics of the catalytic hydroxylation of salicylate and several analogues. We also show that 1-hydroxy-2-naphthoate is not a substrate but triggers NADH oxidation at a high rate in a totally uncoupled reaction. This reaction might inhibit PAH degradation by sphingomonads when phenanthrene is an abundant substrate.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Strains of *Escherichia coli* and *Pseudomonas putida*, and plasmids used for gene overexpression, as well as general culture conditions, have been previously described (9). Plasmid pVEHD11 carrying the *phnA1bA2b* coding sequence fused to a 5'-extension coding for a Histag, was constructed in two steps. A NdeI-BamHI fragment carrying the two genes of interest was isolated from plasmid pDAB11 (9) and subcloned into pET15b (Novagen) to give pEHD11. The insert was then extracted from pEHD11 as a XbaI fragment and cloned into pVLT31 (8). A plasmid carrying *phnA1bA2b* in the right orientation with respect to the P_{tac} promoter was selected and designated pVEHD11. Overproduction of ht-PhnII was

performed either in *E. coli* BL21(DE3) carrying pEHD11 or in *P. putida* KT2442 carrying pVEHD11. Overproduction of ht-PhnA3, ht-PhnA4 and ht-Red_{B356} that were used as electron carriers for ht-PhnII in oxygenase assays has been previously described (13).

Large-scale cultures of strain KT2442(pVEHD11) were grown on Luria-Bertani rich medium in a 12-L fermentor as previously described (13). Expression of the recombinant oxygenase was induced at 25°C with 0.2 mM IPTG, and incubation was prolonged for 20 h before harvesting the culture by centrifugation. The bacterial pellet was washed with 50 mM Tris-HCl buffer (pH 7.5) and kept frozen until use.

Purification of the oxygenase component PhnII

All purification procedures were carried out under argon, using buffers equilibrated for at least 24 h in a glove box maintained under anoxic conditions (O_2 < 2 ppm, Jacomex, France). The temperature was kept at 0-4 °C except when otherwise indicated. A crude extract was prepared by thawing the bacterial pellet (100 g) in two volumes of lysis buffer (50 mM Tris-HCl, pH 7.5) by volume, followed by lysozyme treatment (0.5 mg/ml) for 15 min at 30°C. The suspension was then subjected to ultrasonication for a total time of 10 min including 5 min of pause at 75% of maximal intensity using a Vibra Cell apparatus run in pulse mode at 5 s/pulse (Fisher Bioblock Scientific, Illkirch, France). The lysate was centrifuged at 12,000 g for 30 min, and the resulting cell extract was diluted two-fold with TG buffer (25 mM Tris-HCl, pH 7.5, containing 5% glycerol and 2 mM β-mercaptoethanol) and applied to two 40-ml columns of DEAE-cellulose (DE52, Whatman) equilibrated with TG buffer. After washing the columns with four bed volumes of the same buffer, the oxygenase was eluted as a brown band with buffered 0.3 M NaCl. The eluate (about 50 ml) was immediately applied to a small column (7 ml) of immobilized metal affinity chromatography (IMAC) resin loaded with Co^{2+} (TALON, BD Biosciences Ozyme, France). The column was washed successively with 8 bed

volumes of TG buffer containing 0.5 M NaCl and 5 bed volumes of the same buffer supplemented with 20 mM imidazole and 1 mM salicylate. A brown protein fraction was then eluted with TG buffer containing 0.15 M imidazole and 1 mM salicylate. A portion of the preparation (1.3 ml containing about 40 mg of protein) was applied to a 1.5 × 60 cm column of Superdex SD200 equilibrated in TG buffer containing 0.1 M NaCl and eluted at a rate of 0.43 ml/min. The purified protein fraction was concentrated onto a small column of DEAE-cellulose, eluted in about 1 ml of TG buffer containing 0.3 M NaCl, and frozen as pellets in liquid nitrogen. This preparation, designated ht-PhnII, was judged to be at least 95% pure by SDS-PAGE (Fig 1).

Purification of other proteins

The ferredoxin (ht-PhnA3) from strain CHY-1 and the reductase component of the biphenyl dioxygenae from *C. testosteroni* B-356 (ht-Red_{B356}) were purified as previously described (13). The catechol 2,3-dioxygenase from *Pseudomonas putida* mt2 (XylE) was purified according to a published procedure (11).

Enzyme assays

Salicylate hydroxylase activity was determined using three different assays. In the coupled assay, the formation of catechol from salicylate was coupled to ring cleavage by catechol 2,3-dioxygenase (XylE). Each reaction mixture contained 0.1 µM ht-PhnII, 1.5 or 3 µM ht-PhnA3, 0.3 µM ht-Red_{B356}, 0.03 µM XylE, 0.2 mM NADH and 0.5 mM salicylate in 50 mM potassium phosphate buffer, pH 7.0. Reactions were carried out at 30°C in quartz cuvettes, and initiated by the injection of a 100-fold concentrated solution of the protein mixture with a gas-tight syringe. The protein mixture was prepared 30 min before starting the assays and kept on ice under argon in phosphate buffer containing 1 mM dithiothreitol, 0.05 mM ferrous

ammonium sulfate and 10% glycerol. The absorption at 376 nm was recorded at 0.1-s intervals over 1 min with a HP8452 spectrophotometer (Agilent Technologies, Les Ulis, France). The enzyme activity was calculated from the initial linear portion of the time course, using an absorption coefficient of 34,000 $M^{-1}.cm^{-1}$ for 2-hydroxymuconate semi-aldehyde (11). Assuming that each molecule of the latter product resulted from the oxidation of one molecule of catechol, one unit of activity was defined as the amount of enzyme that converted one micromole of salicylate to catechol per minute.

The enzyme activity was also measured by recording NADH oxidation at 340 nm, and calculated using an absorbance coefficient of 6,220 $M^{-1}.cm^{-1}$. The protein mixture used had the same composition as that described above, except that XylE was omitted. Alternatively, the oxygenase activity was determined by monitoring O_2 uptake using a Clark-type O_2 electrode under conditions essentially similar to those previously described (13). Assays contained NADH at an initial concentration of 0.4 mM. After approximately 60 nmol O_2 had been consumed, 300 U of liver catalase (Sigma-Aldrich) was added to estimate the amount of H_2O_2 formed during the assay. In some cases, 0.6 ml of reaction mixture was immediately withdrawn, and acidified with 0.1 % acetic acid. After centrifugation and filtration, samples were analyzed by HPLC to quantify the catechols formed as described below.

Steady-state kinetics

Sets of salicylate hydroxylase assays were carried out at substrate concentrations that were varied over a 0.5-100 µM range. Activity was measured in the XylE-coupled assay as described above. All assays were performed in duplicates, and at least 12 concentrations were tested. For titration of the hydroxylase with anthranilate, enzyme activity was measured by monitoring NADH oxidation at 340 nm. Assays were performed for substrate concentrations ranging between 5 and 500 µM. Oxidation rates were calculated using an absorption

coefficient incremented of 0.874 $M^{-1}.cm^{-1}$ to take into account the absorbance of anthranilate at 340 nm. Plots of the initial reaction rate versus substrate concentration were fitted to the Michaelis-Menten equation using the curve fit option of Kaleidagraph (Synergy Software). Only curve fits showing correlation coefficients better than 0.98 were considered.

Chemical identification and quantification of the enzymatic products

The oxidation products generated by PhnII were analyzed by GC-MS. Samples were derivatized with bis(trimethysilyl)trifluoroacetamide : trimethylchlorosilane (99:1) from Supelco (Sigma-Aldrich), prior to analysis using a HP6890 gas chromatograph coupled to a HP5973 mass spectrometer (Agilent Technologies). Operating conditions were as previously described (12), and mass spectrum acquisitions were carried out in the scan mode.

Concentrations of catechol and methylcatechols formed by salicylate 1-hydroxylase were determined by HPLC using a Kontron system equipped with a F430 UV detector. Samples (0.1 ml) were injected onto a 4×150-mm C8 reverse-phase column (Zorbax, Agilent Technologies) run at 0.8 ml/min. The column was eluted with a linear gradient of 0 to 50% acetonitrile in water over 15 min. Detection was carried out at 276 nm. Calibration curves were generated by injecting known amounts of 3-methyl-, 4-methyl- or unsubstituted catechol.

Determination of the iron content of proteins

To extract iron from proteins, samples (150 µl) were treated with 2.5 N HCl for 30 min at 95°C, then diluted with 135 µl of ultrapure water. Iron was assayed in triplicate as a complex with bathophenanthroline by absorbance measurement at 536 nm (2). A calibration curve was generated by diluting a standard solution of ferric nitrate containing 1g/L Fe (Merck).

Protein analyses

Routine protein determinations were performed using the Bradford assay (4), or the bicinchoninic acid reagent kit (Pierce) using bovine serum albumin as a standard. A modified microbiuret assay was used for measuring the protein content of purified preparations of ht-PhnA3 and ht-PhnII (19). SDS-PAGE on mini-slab gels was performed as previously described (14). The molecular mass of purified ht-PhnII was determined by size-exclusion chromatography on a column (1.5 x 60 cm) of Superdex SD200 (Amersham Biosciences). The column was run at a flow rate of 0.2 ml/min and calibrated with the following protein markers: Ferritin (443 kDa), catalase (240 kDa), aldolase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and myoglobin (17 kDa), all from Sigma-Aldrich.

EPR analysis

Purified ht-PhnII was adjusted to 30 μM in 25 mM Tris-HCl, pH 7.5 containing 5% glycerol and reduced with 1 mM dithionite. The EPR spectrum was recorded at 4 K as previously described (13).

RESULTS

Purification and biochemical properties of salicylate 1-hydroxylase from strain CHY-1

The structural genes encoding the hydroxylase were cloned into pET15b in order to produce the enzyme with a His-tag fused at the N-terminus of the large subunit. When introduced into an appropriate *E. coli* overexpression strain, two polypeptides of the expected sizes were detected in the cell extract by SDS-PAGE, but the enzyme was essentially inactive. Transfer of the genes into plasmid pVLT31 under the P_{tac} promoter resulted in an efficient expression system producing active enzyme in both *E. coli* and *P. putida*. The latter organism was preferred as a host to overproduce the recombinant enzyme because it consistently afforded better purification yields compared to *E. coli*. The hydroxylase was purified under anoxic conditions using a three-step procedure as described under Materials and Methods, yielding a protein preparation that was judged more than 95 % pure based on SDS-PAGE (Fig. 1). It consisted of two polypeptides with apparent M_r of 47,000 and 21,000. We observed that the enzyme activity fell dramatically upon IMAC chromatography, most likely because the enzyme lost a good part of its active site Fe(II) ion. This loss of activity could be prevented by supplementing buffers with 1 mM salicylate, while ethanol or isopropanol had no protecting effect. After the last purification step, maximal activity was obtained by incubating the protein preparation for 1 h at 4°C with 0.1 mM ferrous ammonium sulfate. The purified enzyme exhibited a specific activity of 0.89 U/mg in a coupled assay in which the catechol formed by the hydroxylase was oxidized to 2-hydroxymuconic acid semialdehyde by catechol dioxygenase (see Methods). The molecular mass of the hydroxylase as deduced from gel filtration experiments was 190 kDa, consistent with an $\alpha_3\beta_3$ hexameric structure. The enzyme exhibited a broad absorbance maximum at 456 nm with a shoulder around 530 nm, which is typical for proteins containing Rieske-type [2Fe-2S] clusters. The occurrence of such clusters in the protein was confirmed by EPR analysis of a dithionite-reduced sample, which gave a

rhombic signal centered around g = 1.91 (data not shown). The iron content of the enzyme was calculated to be 8.52 ± 0.21 Fe/mol based on chemical assay, which is close to the theoretical value for an $\alpha_3\beta_3$ hexamer containing one [2Fe-2S] cluster and one non-heme iron per α subunit. Overall, the salicylate hydroxylase from strain CHY-1 exhibited biochemical properties similar to those of classical Rieske-type dioxygenases that catalyze the dihydroxylation of various aromatic hydrocarbons (5).

Catalytic properties and substrate specificity

The activity of the purified ht-PhnII oxygenase required ancillary proteins PhnA4 and PhnA3 to transfer electrons derived from NADH oxidation to the enzyme active site. The dependence of the enzyme complex activity on the concentration of each electron carrier was studied in assays where their molar ratio relative to the oxygenase component was independently varied. While half-saturation was reached at a reductase-to-oxygenase ratio close to 0.5, a much higher relative concentration of ferredoxin ht-PhnA3 was needed to reach maximum activity. At the highest protein ratio tested (about 200-fold molar excess of ferredoxin), the enzyme activity was not fully saturated. Nevertheless, in the assays described herein this ratio was set at 30 or below, in order to prevent excessive substrate-independent NADH oxidation by the enzyme complex. Also, the reductase component of the biphenyl dioxygenase from *Comamonas testosteroni*, ht-RED_{B356}, was substituted for PhnA4 in most assays because the reductase from strain CHY-1 proved to be an unstable protein (13). No loss of hydroxylase activity was observed as a result of this replacement.

The substrate specificity of the hydroxylase toward various salicylate analogues was first determined by NADH oxidation assays (Table 1). At the end of each assay, the product formed was extracted with ethyl acetate, and analyzed by GC-MS. The enzyme showed high levels of activity with all the methylsalicylates tested, with a preference for 4-

methylsalicylate. Analogues bearing a substituent with a high electron withdrawing effect, like 5-chloro- and 5-nitrosalicylate were poor substrates. 2,4- and 2,6-Dihydroxybenzoates were converted to products identified as trihydroxybenzenes by GC-MS, but gentisate (2,5-dihydroxybenzoate) gave no detectable product. These three compounds yielded relatively small amounts of product (if any), which did not correlate with the high NADH oxidation rates observed, indicating that the catalytic reaction was uncoupled. Anthranilate also gave rise to substantial NADH oxidation while producing little 2-aminophenol as the only detectable product. When 1-hydroxy-2-naphthoate and 2-hydroxy-1-naphthoate were provided as substrates, NADH oxidation was the highest but no product was formed, suggesting that the reaction was fully uncoupled. To further investigate the catalytic properties of strain CHY-1 salicylate 1-hydroxylase, enzyme reactions were monitored by amperometric measurements of O_2 uptake.

Uncoupled reactions catalyzed by salicylate 1-hydroxylase

Substrate-dependent oxygen uptake by CHY-1 hydroxylase as well as hydrogen peroxide formation during the assays were measured for several salicylate analogues (Table 2). When possible, catechol products were quantified in order to determine the coupling efficiency between oxygen consumption and catechol formation. A fairly good coupling was observed upon enzymatic oxidation of salicylate and methylsalicylates, although a small proportion of the O_2 consumed was reduced to H_2O_2. As the enzyme also formed hydrogen peroxide in the absence of substrate (data not shown), we assume that this low activity might result from an autooxidation of the ht-PhnA3 ferredoxin, as previously observed with a similar enzyme system (13). When anthranilate and nitrosalicylate were used as substrates, a greater proportion of the oxygen consumed was converted to peroxide. The two hydroxynaphthoate molecules gave rise to high rates of oxygen uptake with no product formation. HPLC

measurements at the end of the reaction revealed that the substrate concentration had not significantly decreased. These results indicated that both hydroxynaphthoates triggered a highly uncoupled enzymatic reaction, as confirmed by the high proportion of oxygen recovered as hydrogen peroxide. Some of the reactions catalyzed by the hydroxylase from strain CHY-1 are illustrated in figure 2.

Steady-state kinetics

Steady-state rates of salicylate hydroxylation by the purified enzyme were measured at substrate concentrations in the range 0.5 to 100 µM. The XylE-coupled assay was used to monitor the reaction because it directly measured the rate of product formation, whereas other assays might have introduced a bias due to the imperfect coupling of the reaction. The enzyme titration gave data that could be fitted by the Michaelis function, from which an apparent K_m of 1.10 ± 0.2 µM was calculated. This value remained constant when the ht-PhnA3/ht-PhnII molar ratio was varied. On the other hand, the apparent turnover number of the hydroxylase was found to depend on this ratio, and to vary from one enzyme preparation to another, probably as a function of the iron content of the enzyme. Catalytic velocity ranged between 0.73 ± 0.04 s^{-1} at a ratio of 15 for one preparation, and 2.9 s^{-1} at a ratio of 30 for another preparation. Inhibition of the hydroxylase activity was observed at high salicylate concentration, reaching 30% at 0.5 mM and 51% at 1 mM. The kinetic parameters of the enzyme towards anthranilate were also examined at substrate concentrations that were varied between 5 and 400 µM. Steady-state rates were determined using NADH oxidation assays. PhnII exhibited an almost 30-fold higher K_m (28 ± 6 µM) for this substrate compared to salicylate, whereas its V_{max} taken as the NADH oxidation rate, was very similar for both substrates. However, since NADH oxidation was poorly coupled to anthranilate

hydroxylation (Table 2), the actual rate of substrate hydroxylation was lower in the case of anthranilate.

Inhibition of salicylate hydroxylation by 1-hydroxy 2-naphthoate

The bacterial degradation of phenanthrene leads to the intermediate metabolite 1-hydroxy 2-naphthoate, which in gram-negative species like *Pseudomonas* sp., is thought to be converted to 1,2-dihydroxynaphthalene by the same enzyme that hydroxylates salicylate (1). Since 1-hydroxy 2-naphthoate was not a substrate of the hydroxylase studied herein, and caused uncoupling of the reaction, we examined whether this compound would inhibit salicylate hydroxylation *in vitro*. To this end, we performed salicylate titration curves at various concentrations of 1-hydroxy 2-naphthoate (Fig. 3A). Results showed that the hydroxylation reaction was strongly inhibited through a mechanism that appeared to be competitive. A plot of K_m /V_{max} values versus inhibitor concentration gave a straight line, which allowed to calculate an inhibition constant of 0.68 μM (Fig. 3B).

DISCUSSION

In this work, we describe the first characterization of a three-component salicylate 1-hydroxylase, an enzyme that appears to be present only in PAH-degrading strains of the sphingomonad group. The oxygenase component of the enzyme exhibited biochemical properties and a co-factor content very similar to that of typical ring-hydroxylating dioxygenases catalyzing the dihydroxylation of various aromatic substrates (5). In addition, the PhnII protein shows significant sequence similarity with anthranilate dioxygenase from *Burkholderia cepacia* DBO1 (52% and 38% identity for the α and β subunits, respectively; (6)), which converts anthranilate to catechol. Nevertheless, PhnII exclusively catalyzes monohydroxylations as exemplified by the conversion of anthranilate to 2-aminophenol.

Phylogenetic analyses have previously shown that the oxygenase components of anthranilate dioxygenase from strain DBO1 and of salicylate 1-hydroxylases from sphingomonads (including PhnII) form a distinct group of enzymes, together with halobenzoate dioxygenases and salicylate 5-hydroxylase (6). Curiously, this group includes enzymes that function only as dioxygenases on the one hand, or as monohydroxylases on the other, and which all use substituted benzoates as substrates. This observation raises questions about the structural features that determine whether an enzyme functions as a mono- or a dioxygenase. Such a question might be addressed in experiments where a monohydroxylase is modified to acquire the ability to catalyze dihydroxylations through site-directed mutagenesis of residues predicted to be at the enzyme active site.

In *in vitro* assays, the catalytic activity of the hydroxylase was highly dependent on the molar ratio of the ferredoxin (PhnA3) and oxygenase (PhnII) components, half-saturation of the activity occurring for an approx. 200-fold excess of PhnA3. A similar observation was made with other three-component oxygenases, including the naphthalene dioxygenase (PhnI) from strain CHY-1 (13). However, in the latter case, half-saturation was achieved with a 14-fold excess of PhnA3, suggesting that the ferredoxin has a different affinity for the PhnI and PhnII oxygenases. This difference might have physiological significance inasmuch as the two oxygenases might compete for the same ferredoxin *in vivo*. Indeed, a single ferredoxin-encoding gene homologous to *phnA3* was identified in the catabolic cluster responsible for aromatic hydrocarbon degradation in three sphingomonads (15, 20, 23). *In vivo*, the ferredoxin requirement is certainly not as high as *in vitro*, because the high protein concentration of the cytosolic compartment probably favors ferredoxin oxygenase interactions.

The three-component salicylate hydroxylase from strain CHY-1 has a low apparent Michaelis constant for its substrate, similar to that reported for counterparts of the flavoprotein-type

found in *Pseudomonas* species (1.72-1.9 μM; (1, 29)). The specific activity of the two types of enzymes is also of the same order (0.89 versus 0.27 U/mg for the *P.putida* enzyme; (1)), although direct comparison is difficult because the *in vitro* activity of the CHY-1 enzyme is strongly dependent on the ferredoxin/oxygenase molar ratio. Ht-PhnII appeared to be almost as active on methylsalicylates as on salicylate. This result is consistent with our finding that the aromatic ring-hydroxylating dioxygenase responsible for the initial attack on PAHs in CHY-1 can oxidize several methyl- and dimethylnaphthalenes (unpublished results), which are then predicted to be further oxidized to methylsalicylates. Ht-PhnII also hydroxylated salicylates with chlorine, nitro and hydroxyl substituents although at slower rates. A broad substrate specificity was also observed for the two flavoprotein hydroxylases from *Pseudomonas stutzeri* AN10 (3) and the three-component hydroxylases from *Sphingobium* P2 (22). However, enzyme activity appeared to be dependent on the nature of the substituent group with an order of preference as follows : CH_3 > Cl > NO_3 > OH. This can be clearly seen by comparing the hydroxylase activity of ht-PhnII on salicylate analogues bearing either one of these groups in the C5 position (Table 1). In this respect, the 5-hydroxysalicylate or gentisate was not oxidized by ht-PhnII, whereas the isomers bearing an hydroxyl in the C4 or C6 position were hydroxylated. This finding indicates that a polar substituent in the C5 position of the ring prevents productive interaction of the substrate at the enzyme active site, possibly because the substrate binding pocket has a hydrophobic character that hinders access of gentisate. Anthranilate was a poor substrate for ht-PhnII as indicated by the uncoupling of the hydroxylation reaction and the relatively high K_m of the enzyme for this substrate. In comparison, the K_m of anthranilate dioxygenase for anthranilate has been estimated to be about 1 μM (10).

The hydroxylation of anthranilate, as did that of other poor substrates, gave rise to a significant uncoupling of the reaction resulting in the formation of hydrogen peroxide. Such a

side reaction has been observed for enzymes with similar structures that function as dioxygenases (13, 17) and seems to be a consequence of the catalytic mechanism of this type of enzyme (27, 28). Indeed, it has been observed that in naphthalene dioxygenase, substrate binding to the active site increases the affinity of the enzyme for oxygen (28). Such a mechanism might explain the high rate of substrate-induced reduction of oxygen to peroxide catalyzed by PhnII in the presence of pseudo-substrates such as hydroxynaphthoate. Interestingly, salicylate hydroxylases of the flavoenzyme type, which have a different catalytic mechanism, were also found to catalyze uncoupled reactions when incubated with certain salicylate analogues such as benzoate (24).

Unlike the flavoprotein hydroxylase from *P. putida* (1), the enzyme from CHY-1 was unable to hydroxylate 1-hydroxy-2-naphthoate, a metabolite that is produced in the catabolic pathway of phenanthrene. Moreover, we bring evidence that this metabolite causes inhibition of salicylate hydroxylation, which might be deleterious to bacterial cells growing on PAHs. Indeed, salicylate is an intermediate in the degradation of several PAHs including naphthalene, anthracene, phenanthrene and fluorene, and its hydroxylation might be a bottleneck if subjected to metabolic inhibition. Such an inhibition could be circumvented *in vivo* if an enzyme oxidizes 1-hydroxy 2-naphthoate and maintains its concentration to a level low enough to prevent inhibition of PhnII, or if another salicylate hydroxylase functionally substitutes for PhnII. In the phenanthrene-degrading strain *Sphingobium* P2, three isoenzymes have been shown to catalyze salicylate hydroxylation (22) and counterparts were identified in two other sphingomonads from the analysis of catabolic genes (15, 23). In *S. yanoikuyae* B1, the hydroxylase encoded by *bphA1cA2c*, which shares high sequence similarities with PhnII (α and β subunits show 79% and 66% identities, respectively) has been proposed to catalyze hydroxylation of both salicylate and 1-hydroxy 2-naphthoate, as deduced from the analysis of a *bphA1c* knockout mutant strain (7). This mutant lost the ability to grow on salicylate and

naphthalene, and accumulated 1-hydroxy 2-naphthoate when incubated with phenanthrene. However, the ability of the enzyme to catalyze the conversion of 1-hydroxy 2-naphthoate to 1,2-dihydroxynaphthalene has not been clearly demonstrated. Besides, the latter study indicates that the *bphA1c* product is required for hydroxylation of salicylate, implying that this enzyme could not be replaced by anyone of the two other isoenzymes present in the bacterium. Hence, the physiological function and raison d'être of multiple copies of three-component hydroxylases in the catabolism of PAHs by sphingomonads remain to be elucidated. Also, further work is needed to identify unambiguously in sphingomonads the enzyme responsible for the hydroxylation of 1-hydroxy 2-naphthoate, which is an essential step in phenanthrene catabolism.

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TABLE 1 : Substrate specificity of ht-PhnII in the NADH oxidation assay and GC-MS analysis of the products formed

Substrate [a]	Enzyme activity [b]		Products detected by GC-MS		Identification of product
	$U.mg^{-1}$	%	RT (min)	Molecular mass	
Salicylate	0.578	100	8.87	254	catechol
3-Methyl-salicylate	0.44	76	9.86	268	3-methyl catechol
4-Methyl-salicylate	0.625	108	9.74	268	4-methyl catechol
5-Methyl-salicylate	0.396	68.5	9.74	268	4-methyl catechol
5-Chloro-salicylate	0.267	46	10.79	288	4-chloro catechol
5-Nitro-salicylate	0.184	32	13.39	299	4-nitro catechol
Anthranilate	0.39	67	10.21	253	2-aminophenol
3,5-dichloro-salicylate	ND[d]	ND[d]	12.14	322	3,5-dichlorocatechol
2,4-dihydroxybenzoate	*1.38*	*184*	11.98	342	Trihydroxybenzene
2,6-dihydroxybenzoate	*0.326*	*44*	11.34	342	Trihydroxybenzene
Gentisate	*1.21*	*162*	-	-	no product
1-hydroxy-2-naphthoate	*1.35*	*179*	-	-	no product
2-hydroxy-1-naphthoate	*10.7*	*1430*	-	-	no product

[a] Initial substrate concentration was 0.5 mM except for 1-hydroxy 2-naphthoate, 2-hydroxy 1-naphthoate (0.25 mM) and 5-nitrosalicylate (0.125 mM).

[b] Activities, expressed as μmol NADH.min^{-1}.mg^{-1}, are means of duplicate assays. Standard deviations are less than 5%. Values in italics are from a different set of assays and refer to an enzyme activity of 0.75 U.mg^{-1} with salicylate as substrate

[c] Products were extracted with ethyl acetate after 2 min of reaction, and analyzed as trimethylsilyl derivates.

Table 2 : Substrate-dependent O_2 uptake, product formation and H_2O_2 release in PhnII-catalyzed reactions

Substrate	O_2 uptake[a] µmol/min/mg	H_2O_2/O_2 ratio	Catechol produced[b] µmol/min/mg
Salicylate	0.757± 0.007	0.144	0.64 ± 0.04
3-Methylsalicylate	0.711 ± 0.010	0.23	0.50 ± 0.01
5-Methylsalicylate	0.659 ± 0.005	0.19	0.61 ± 0.01
5-Chlorosalicylate	0.630 ± 0.025	0.19	nd
5-Nitrosalicylate	0.416 ± 0.005	0.38	nd
Anthranilate	0.630 ± 0.02	0.42	nd
1-hydroxy-2-naphthoate	3.07 ± 0.01	0.70	No product
2-hydroxy-1-naphthoate	8.56 ± 0.01	0.65	No product

[a] Maximum velocities calculated from the initial O_2 uptake rate taken during the first minute of the time course. The PhnA3/PhnII molar ratio was 30.

[b] As determined from HPLC quantification after about 3 min. nd means not determined because no appropriate standard was available to quantify the relevant products.

Figure legends

Figure 1 : SDS-PAGE protein analysis during purification of recombinant ht-PhnII from strain KT2442 carrying pVEHD11. Lane 1, protein markers; lane 2, soluble cell-free extract; lane 3, DEAE column eluate; lane 4, IMAC column eluate; lane 5, dialyzed purified protein.

Arrows point to the α and β subunits of ht-PhnII.

Figure 2 : Reactions catalyzed by the three-component hydroxylase from strain CHY-1.

The scheme shows reaction products formed from (a) methylsalicylate, (b) anthranilate and (c) the pseudosubstrate 1-hydroy 2-naphthoate.

Figure 3 : Inhibition of salicylate hydroxylation by 1-hydroxy 2-naphthoate.

A) Steady state rates of salicylate hydroxylation were measured in the presence of 1-hydroxy 2-naphthoate at the following concentrations (μM): 0 (diamonds), 0.8 (filled circles), 2 (triangles), 5 (inversed triangles), 8 (open circles). Activities were measured in the coupled assay with an enzyme complex containing ht-PhnII and ht-PhnA3 in a molar ratio of 1:15. Michaelis curve fits were adjusted to the data points as described in Methods. B) Plot of K_m/V_{max} versus inhibitor concentration.

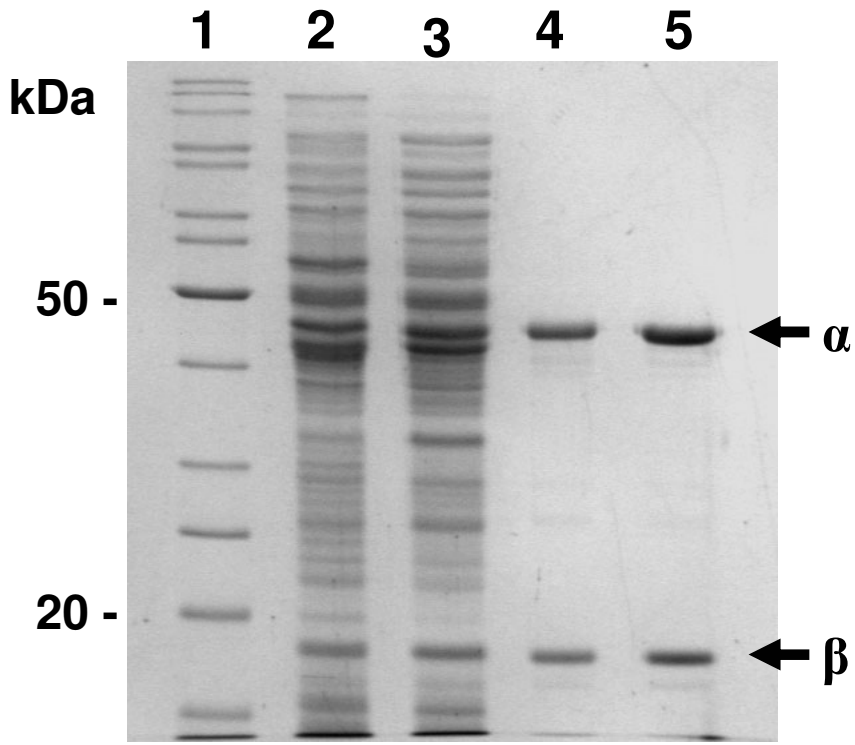


Fig.1, Jouanneau et al., 2007

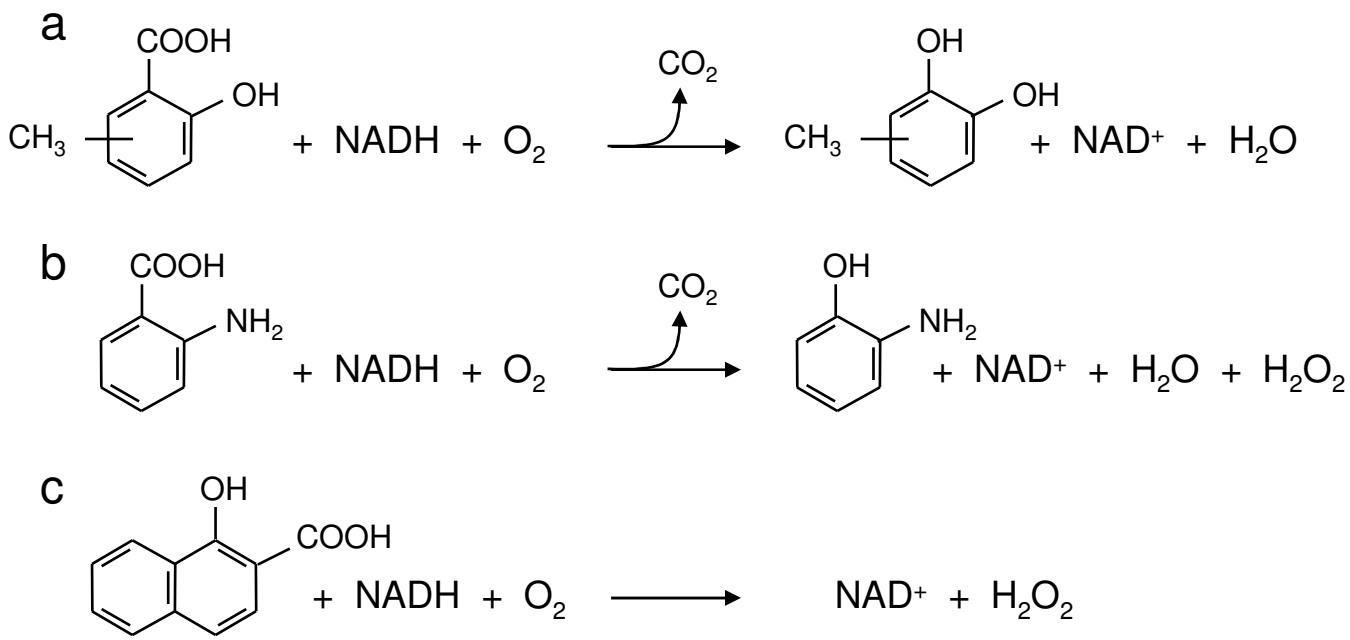
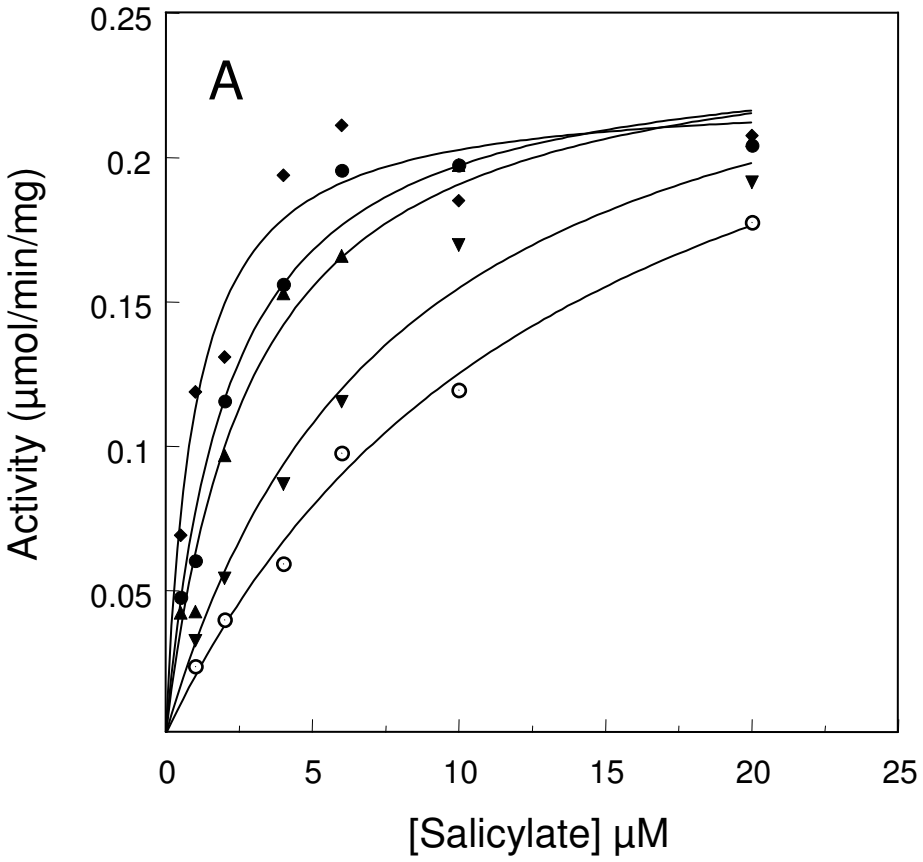


Fig. 2, Jouanneau et al.



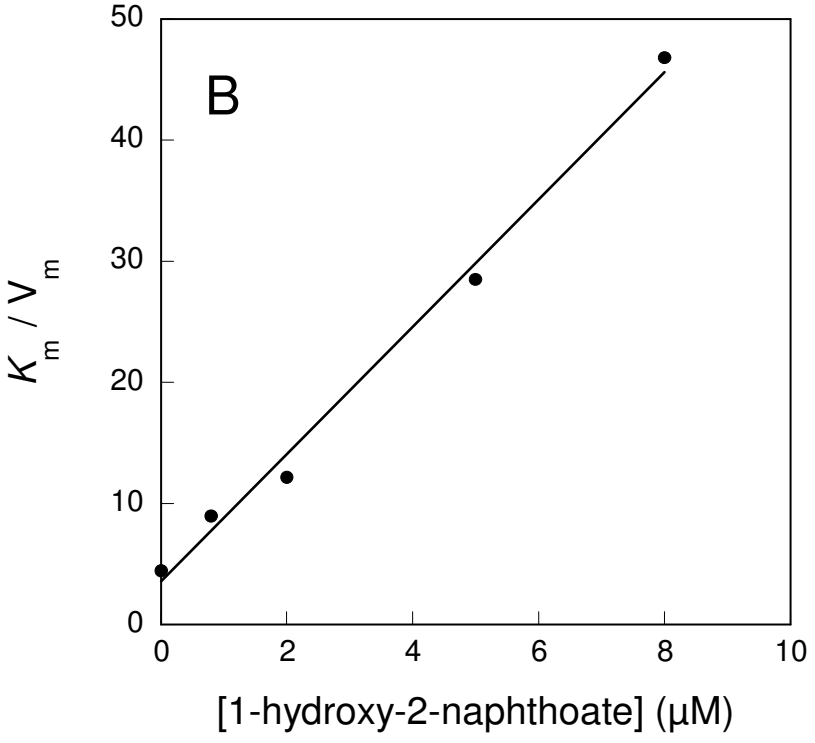


Fig. 3, Jouanneau et al., 2007